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CONTRACT NO: DAMD17-87-C-7061

TITLE: DEVELOPMENT OF DENGUE VACCINE, PHASE I

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REPORT DATE: July 1, 1987

TYPE OF REPORT: Final, Phase I

PREPARED FOR: U.S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND
Fort Detrick, Frederick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for public release;
distribution unlimited

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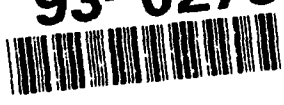

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1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	July 1, 1987	Final, Phase I (12/15/86-6/15/87)

4. TITLE AND SUBTITLE

Development of Dengue Vaccine, Phase I

5. FUNDING NUMBERS

Contract No.
DAMD17-87-C-7061

65502A
3P665502M802.AA.042
WUDA312787

6. AUTHOR(S)

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9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research & Development Command
Fort Detrick
Frederick, Maryland 21702-5012

10. SPONSORING/MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION/AVAILABILITY STATEMENT

Approved for public release; distribution unlimited

12b. DISTRIBUTION CODE

Dengue virus is a mosquito borne, positive strand RNA virus responsible for hundreds of thousands of illnesses annually. No effective vaccine exists for any of the four dengue serotypes. The ultimate goal of our research is the production of a vaccine for dengue type 2. As a preliminary step toward this goal, we have cloned randomly primed cDNA fragments of dengue type 2 in the bacterial expression vector, λ gt11. When cloned in the proper translational orientation and reading frame, the recombinant clones will express β-galactosidase-DEN2 fusion proteins. Recombinants expressing such fusion proteins can be detected immunologically. As judged by the frequency of interruption of the β-galactosidase gene, approximately 25% of the vectors contain inserts.

14. SUBJECT TERMS

Dengue type 2 virus, cDNA expression library, vaccine development, SBIR, Phase I, RAD I

15. NUMBER OF PAGES

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

APPENDIX B
DOD No. 88.1

U.S. DEPARTMENT OF DEFENSE

SMALL BUSINESS INNOVATION RESEARCH PROGRAM
PHASE 1 — FY 1988
PROJECT SUMMARY

Topic No. A86-209 | Military Department/Agency U.S. Army

Contract No. DAMD 17-87-C-7061 | Medical Research and Development Command

Name and Address of Proposing Small Business Firm
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Title Proposed by Small Business Firm

Development of a Dengue Vaccine, Phase I

Technical Abstract (Limit your abstract to 200 words with no classified or proprietary information/data.)

Dengue virus is a mosquito borne, positive strand RNA virus responsible for hundreds of thousands of illnesses annually. No effective vaccine exists for any of the four dengue serotypes. The ultimate goal of our research is the production of a vaccine for dengue type 2. As a preliminary step toward this goal, we have cloned randomly primed cDNA fragments of dengue type 2 in the bacterial expression vector, λgt11. When cloned in the proper translational orientation and reading frame, the recombinant clones will express β-galactosidase-DEN2 fusion proteins. Recombinants expressing such fusion proteins can be detected immunologically. As judged by the frequency of interruption of the β-galactosidase gene, approximately 25% of the vectors contain inserts.

Anticipated Benefits/Potential Commercial Applications of the Research or Development

Screening of the cDNA expression library with human convalescent antisera will allow identification of recombinants expressing DEN2 antigenic determinants as fusion proteins, and β-galactosidase affinity columns will allow purification of large amounts of fusion proteins. DNA sequencing of the clones and immunological characterization of the fusion proteins will allow identification of dengue peptides involved in eliciting an immune response. Based on this information, development of a dengue vaccine could proceed.

List a maximum of 8 Key Words that describe the Project.

Dengue type 2 virus, cDNA expression library, vaccine development

Hawaii Biotechnology Group, Inc.

John M. Ivy, Ph.D. 312-58-6053

Development of a Dengue Vaccine, Phase I

Final Report

INTRODUCTION

Dengue virus is a single stranded, mosquito borne RNA virus. When measured in terms of global morbidity, the four serotypes of dengue virus are the most important viruses transmitted to man by arthropods. It is responsible annually for hundreds of thousands of illnesses world wide in the tropics. In cases of severe reaction to the virus, illness is characterized by hemorrhagic fever or dengue shock syndrome. To date, no effective and safe vaccine against any of the four dengue serotypes has been developed. To begin development of a vaccine for dengue type 2 (DEN2), we have proposed to construct a cDNA library of the viral genome. This library was made of random cDNA fragments cloned in the expression vector λgt11 (Young & Davis, 1983). This vector, when induced, expresses inserts cloned in the proper translational orientation and reading frame as fusion proteins with the E. coli enzyme beta-galactosidase. Such a library may be screened immunologically to detect recombinants expressing DEN2 antigenic determinants. This report describes the construction of such a library and our progress to date in characterizing it.

MATERIALS AND METHODS

DENGUE VIRUS. Dengue type 2 (New Guinea, 1944) was cultured in the laboratory of Dr. Leon Rosen (Arbovirus Program, Leahi Hospital, Honolulu, HI) on Aedes albopictus C6/36 cells in tissue culture. Culture supernatant was provided to us frozen (-70°C), and contained typically 10^9 infectious units per ml.

VIRUS PURIFICATION. Virus was purified by either of two methods. Either a crude preparation was made by polyethylene glycol (PEG) precipitation, or the virus was purified further by gradient centrifugation (Obijeski et al, 1976, with modifications suggested by Dr. R. Kinney, Division of Vector-Borne Viral Diseases, Centers for Disease Control, Fort Collins, CO). Cell culture supernatants were clarified by a 30 min. centrifugation at 10,000xg, after which 7 g PEG per 100 ml was added and slowly stirred for 3 h. at 4°C. A PEG precipitate containing virus was collected by centrifugation at 10,000xg for 30 min. at 4°C. For further purification, the precipitate was resuspended in about 1 ml of TNE buffer (0.01 M Tris, pH 8.5, 0.15 M NaCl, 0.001 M EDTA) and layered onto a continuous 30% glycerol, 45% potassium tartrate (in TNE) gradient (Obijeski et al, 1974). This gradient was centrifuged at least 3.5 h at 40,000 rpm in an SW41 rotor at 4°C. The virus band was collected, diluted at least two fold with TNE, and loaded onto a second glycerol, potassium tartrate gradient. This gradient was centrifuged at 40,000 rpm overnight, the virus band collected and diluted at least two fold, and virus was pelleted through a 30% glycerol cushion using an SW41 rotor, 40,000 rpm, 4°C, 4 h. The resulting virus pellet was resuspended in 0.5 ml TNE buffer.

RNA ISOLATION. RNA was isolated from purified virus by proteinase K, SDS treatment followed by phenol-chloroform and chloroform extraction. Proteinase K was added to a final concentration of 10 ug/ml and incubated at 37°C for 30 min. SDS was then added to a final concentration of 1% and incubated an additional 30 min. at 37°C. Proteins were removed by extraction with phenol-chloroform (phenol:chloroform:isoamyl alcohol, 25:24:1) followed by chloroform (chloroform:isoamyl, 24:1) extraction, and RNA was precipitated by adding NaAc to 0.3M plus 2.5 volumes of cold ethanol. All plasticware for RNA work had been siliconized and treated with 0.1% diethylpyrocarbonate to destroy RNases.

cDNA SYNTHESIS. cDNA synthesis made use of a kit purchased from Bethesda Research Laboratories. A detailed description of the protocol can be found in their cDNA synthesis manual (cat. no. 8267SA). We used random hexanucleotides (Pharmacia) generated from calf thymus DNA by DNaseI digestion as primer (Binns et al, 1985). In brief, first strand cDNA was synthesized using Moloney murine leukemia virus reverse transcriptase, and second strand was synthesized with DNA polymerase I and RNase H (Gubler and Hoffman, 1983). In this method, second strand is primed by oligoribonucleotides generated by the nicking action of the reverse transcriptase. An aliquot of the first strand and the entire second strand reactions were labeled with α-^{32}PdCTP, and these radiolabled nucleotides were separated on an alkaline agarose gel to determine the size distribution of cDNAs.

CLONING. Enzymes used in cloning were purchased from a variety of suppliers (Bethesda Research Laboratories, New England Biologicals, International Biotechnology Inc., Boehringer Mannheim, Pharmacia) and were used in accordance with the directions of the manufacturer. Cloning of cDNA made use of the lambda expression vector λgt11 (Huynh et al, 1985), which has an EcoRI cloning site. To prepare cDNA fragments for cloning, their ends were made blunt by the action of T4 DNA polymerase. Twelve nucleotide EcoRI linkers (Boehringer Mannheim) were then added using DNA ligase. These linkers are unphosphorylated and result in the ligation of a single strand only, after which the nonligated strand may be melted off exposing an EcoRI sticky end (Seth, 1984). The ligated linker single strand was phosphorylated using ATP and polynucleotide kinase, and the modified cDNAs were ligated to EcoRI cut, dephosphorylated λgt11 arms (Promega Biotech).

Lambda phage DNA was packaged into phage particles *in vitro* using *E. coli* packaging extracts (Promega Biotech). Phage particles were plated on *E. coli* strain Y1090(r-), which was incubated at 42°C for 2-4 h. after infection to prevent lysogenization before transfering to 37°C. Cloning at the EcoRI site of λgt11 interrupts the β-galactosidase gene. Interruption of the *lacZ* gene can be assayed by providing the chromogenic

substrate 5-bromo-4-chloro-3-indoyl-β-D-galactoside (X-Gal) and the lactose operon inducer isopropyl-β-D-thiogalactopyranoside (IPTG) in the culture medium. In this way, recombinants with inserts are detected as white plaques, while nonrecombinants are distinguished as blue plaques. Immunoscreening (Hyunh et al, 1985) used reagents from Promega Biotech. Bacterial culture and phage manipulations used conventional techniques as described in Maniatis et al (1982).

RESULTS AND DISCUSSION

The strategy for construction of a DEN2 cDNA library as outlined in the proposal involved generation of random cDNA fragments. The rationale for doing so was to enable immunoscreening with human convalescent antisera to "see" DEN2 epitopes removed from the context of the entire gene product, and to cover the entire genome. Three technical objectives were stated in the Phase I proposal. The first was to develop the use of calf thymus DNA digestion products as primers of cDNA synthesis in order to produce random cDNA fragments. Binns et al (1985) have used DNaseI generated oligonucleotides from calf thymus DNA as cDNA primers for a large RNA virus and found clones to have a random distribution throughout the genome. As shown below, priming cDNA synthesis with hexanucleotides from calf thymus DNA worked well in our hands. The second objective was to employ a technique complementary to the above method. This involved partial digestion of RNA with various RNases, addition of poly(A) tails to the RNA fragments with poly(A) polymerase, and priming cDNA synthesis with oligo-d(T). This technique requires controlled digestion of the RNA and greater quantities of starting material than we had (see below). Thus, we chose not to pursue this technique. The third objective was to clone the duplex cDNA in λgt11. This has been done. Screening for foreign DNA inserts is proceeding.

Virus Purifications and cDNA Syntheses. Dengue 2 (DEN2) virus from one liter of Aedes albopictus culture supernatant was purified sequentially on two glycerol/potassium tartrate gradients, and RNA was extracted. We, however, were unable to detect any RNA based on the OD_{260}. Viral bands on the gradients, visible as opalescent material, had been very faint. Due to the possibility of material loss whenever the virus is manipulated, we decided to minimize manipulations by halting purification of the virus after polyethylene glycol (PEG) precipitation. Thus, from a second liter of mosquito cell culture supernatant we obtained RNA, 0.3 ug based on OD_{260}, albeit a cruder preparation.

Starting with 0.3 ug of DEN2 RNA, cDNA synthesis primed by random oligonucleotides yielded about 30 ng of duplex DNA. Strand size from both the first and second reactions ranged in size from 150 - 500 base pairs. The DEN2 cDNA was cloned as described in materials and methods and plated on indicator bacteria. No recombinant phage were detected, however. Controls for packaging

efficiency were acceptable: 5.6×10^8 plaque forming units (pfu)/ug of wild type λ DNA; and ligation of a control insert with the vector yielded 70 times as many plaques with inserts (8.1×10^6 pfu/ug λDNA) as without inserts.

Virus was purified again from a third liter of culture supernatant by PEG precipitation and the RNA extracted. This yielded 2.3 ug RNA with reasonable purity ($OD_{260}/OD_{280} = 1.6$). To examine the size of the RNA, we added a radiolabeled poly(A) tail using polyA polymerase and α-32P ATP. The products of this reaction when size separated on a formaldehyde gel confirmed the presence of a high molecular weight species and a collection of much smaller molecular weight RNAs. Small RNAs were absent in a control reaction using size standard RNAs; thus, it is possible that the small molecular weight material is present in our DEN2 RNA and is not a product of the polymerase reaction. We reason that the small molecular weight RNAs in our sample should at worst reduce the frequency of cloned cDNAs that are reactive with human convalescent antisera. If the small RNAs are degraded DEN2 RNA, then their cDNAs will not be undesired. If their cDNAs are very small (less than 72 bp), they will be removed by the columns used to remove unincorporated nucleotides. And use of convalescent antisera along with proper negative control serum should not identify recombinants that are not DEN2 in origin.

Starting with 1 ug of the above DEN2 RNA, cDNA was synthesized (see Material and Methods). Analysis of the reaction on an alkaline agarose gel (Fig. 1) indicated that first strand products ranged from less than 150 nucleotides to about 800 nucleotides. There was a peak of material (not visible in Fig. 1) at 500 nucleotides. The products of second strand synthesis ranged to greater lengths. Some of this may be due to fold back priming of second strand synthesis or protein contamination of the sample. Use of random oligonucleotide primers for cDNA synthesis on a control RNA template (contained in the BRL cDNA kit) yielded first and second strand products in the size range 300 - 1000 nucleotides (Fig. 1). EcoRI linkers were added to DEN2 cDNA, which was then ligated to λgt11 and packed *in vitro* into phage particles.

The library-containing phage were plated on *E. coli* on medium containing XGal and IPTG. The frequencies of blue (without insert) and white (with insert) plaques indicated that about 25% of the phage contain inserted DNA. Based on the titer of white plaques, we estimate that the library contains approximatley 5×10^4 unique recombinants. Assuming that one of every six inserts is in the proper translational orientation and reading frame, the library should contain about 8,000 recombinants with β-galactosidase-dengue fusion genes. Given a genome size of about 11,000 nucleotides (Stollar et al, 1966), the expression library has 3' ends frequent enough to occur more than once every two nucleotides of the DEN genome.

The cDNA library has been amplified, and one half has been screened preliminarily with human convalescent antisera. A pool of four sera with high titers (three were about 25,000, one was about 6,000; L. Rosen, personal communication) of anti-DEN2 antibodies as assayed by hemagglutination inhibition was used. Multiple signals were seen with the convalescent antisera, but not with a negative control human serum. Thirty of these were picked, and twelve were plaque purified. These twelve upon rescreening did not yield any positive signals. We do not interpret this result as indicating that the library lacks DEN2 sequences, because the antisera have not been characterized as to their reactivity to DEN proteins on a filter membrane format. This is work originally planned for Phase II and will have to await that time.

SUMMARY AND PROSPECTS

We have made cDNA to DEN2 RNA using random primed synthesis, and have cloned the cDNA in the expression vector λgt11. Preliminary immunoscreening has identified no dengue peptides. The human sera, however, needs to be properly characterized before we repeat this screen and can draw any conclusions. At this time we are examining lambda phage DNA minipreparations from two dozen random white plaques for foreign DNA inserts.

Having a cDNA library in λgt11 will offer many advantages. Sequencing primers for use with this vector are commercially available, which will allow sequencing of DEN2 inserts without the need for subcloning. The nucleotide sequence of the DEN2 structural genes is known (Deubel et al, 1986); thus, we will be able to correlate immunologically reactive clones with specific structural genes once the clones have been sequenced. In addition, cloning of DEN2 sequences as fusion genes with the *E. coli* β-glactosidase gene will allow the isolation of milligram amounts of fusion gene products. Once isolated, these proteins could be used as immunogens, and the antibodies elicited could be screened for viral neutralizing ability.

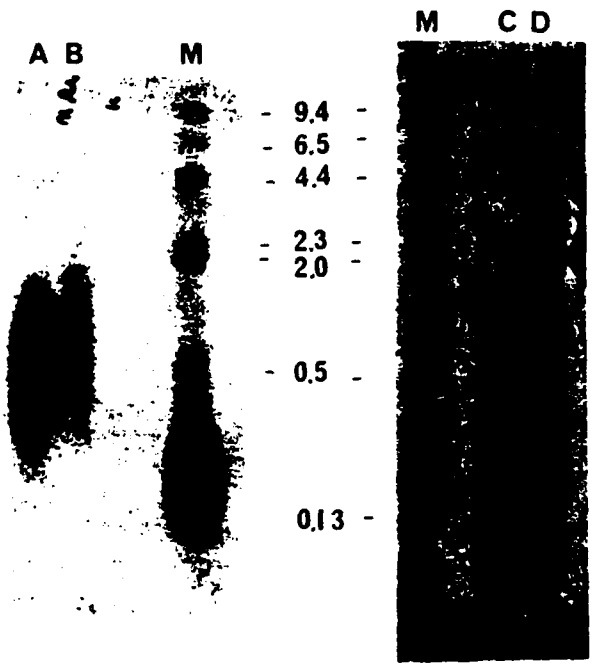

Figure 1. Products of cDNA syntheses.

Lanes A) and B). Products of first and second strand syntheses, respectively, using a 2.3kb control RNA as template and random oligonucleotides as primer. Lanes C) and D). Products of first and second strand syntheses, respectively, using DEN2 RNA as template and random oligonucleotides as primer. Radiolabeled DNA was size fractionated on 0.8% alkaline agarose gels and exposed to X-ray film. Size of marker fragments (in lanes M) are given in thousands of nucleotides.

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